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Genetic diversity of selected *Gladiolus* (*Gladiolus* × *gandavensis* Van Houtte) cultivars assesed by microRNA-based markers

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Gladiolus as economically important flowering plants reflect the wide range of morphological variability of the inflorescences. The aim of current gladiolus breeders is to create interesting new genotypes by different breeding techniques, carrying a variety of colour, size, texture and shapes of flowers and inflorescences. Despite the well-established morphological characterization of gladiolus germplasm, genomic screening by functional markers can provide useful information for breeders. The miRNA-based assay was conducted on 9 gladiolus (*Gladiolus* × *gandavensis* Van Houtte) hybrids by 9 individual markers of following families: miR156, miR160, miR398, miR408, miRr414 and miR482. A total of 291 loci were amplified, of which loci of marker miR408 were the most abundant (25%), with the following representation of other marker types: 21% – miR414; 19% miR156; 16% – miR160; 12% – miR482 and 7% – miR398. Genetic diversity of selected gladiolus cultivars was assessed by two markers, lus-miR408 and hyp-miR414, which provided genotype-specific marker profiling in the form of molecular fingerprinting. Stress-sensitive marker miR398 specifically amplified loci in genotype Správna Susane, which is the most susceptible to water deficit of all analysed genotypes. Marker miR141 was able to distinguish cultivars Athos and Dandy among themselves, but also from other cultivars.

Keywords: DNA fingerprinting, microRNA markers, *Gladiolus* × *gandavensis*

Introduction

Gladiolus is bulbous flowering plant, economically important, cultivated throughout the world for its colourful spikes. The genus *Gladiolus* L. consists of approximately 265 species and is one of the most widespread genera of the family Iridaceae Juss. The center of diversity for the genus *Gladiolus* is considered the Cape of Good Hope, located in the Republic of South Africa. It is widespread throughout the region of tropical Africa, Madagascar, Europe, the Mediterranean, the Arabian Peninsula, Asia, including Afghanistan and Iran (Kumar et al., 2016; Singh et al., 2016). The most widespread is the hybrid gladiolus *Gladiolus* × *gandavensis* Van Houtte). *Gladiolus* varieties exhibit a huge rang of variability in colour, size, texture and shape of flowers, growth habit and flowering behaviour (Singh et al., 2017).

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The aim of the current gladiolus breeders is to create, by different breeding techniques, interesting new genotypes bearing a variety of colours, sizes, textures, as well as a variety of sizes and shapes of flowers (Chaudhary et al., 2018). *Gladiolus* are alien-pollinating species showing diverse pollination mechanisms (Singh et al., 2018). Polyploidy and hybridization have played a significant part in evolution of gladioles. The genus has a basic chromosome set of 15 (n = 15), but overall, the species range from diploids (2n = 30) to hypododecaploid (2n = 180) (Chaudhary et al., 2018; Singh et al., 2018). *Gladiolus* have the smallest genome size of a number of ornamental species of bulbous and tuberous plants. Their genome reaches the size of 1100 Mbp (mega base pairs) for the haploid genome (n).

Gladiolus have a high coefficient, both vegetative using coral – brut, (capable of producing over 500 corals during one vegetative period) and generative reproduction (capable of producing over 500 seeds from a single plant during vegetation) (Pathania and Misra, 2003). Most of the commercial varieties of gladiolus over the past decades have been created using the hybrid crossing technique. Although the mutation through physical or chemical mutagens has been an effective means used to obtain new varieties. The aim of the induced mutagenesis was to create new commercially interesting varieties with fragrance, more complex colouring of petals, full bloom, distinctive spotted drawing of floral petals, more pronounced border at the edges of petals and veining of the leaves (Pathania and Misra, 2003; Kasumi, 2005).

Although there are not many theoretical foundations regarding the genetic characteristics of gladiolus, genomic analyses of this species could be useful to breeders. Molecular markers are an important tool for the analysis of plant genomes and genetic variability.

The most applied molecular markers used for the evaluation of genetic diversity and population structure of gladioulus are ISSR (Inter Simple Sequence Repeats), DAMD (Directed Amplification of Minisatellites) and RAPD-derived SCARS (Random Amplified Polymorphic DNA-derived Sequence-Characterised Amplified Region) markers (Kumar et al., 2016; Singh et al., 2016, 2017a, 2017b, 2018; Chaudhary et al., 2018).

RNA Based Markers – RBMs represent functional type of molecular markers used in genetic technologies (Bežo et al., 2015). An ideal molecular marker is highly polymorphic, occurring in different forms that are different from each other (Gálová et al., 2018). Molecular markers developed based on miRNA molecules represent a new, low cost, protocol transferable, reproducible, stable, and highly efficient process of genotyping and assessing the genetic potential of plants (Fu et al., 2013; Mondal and Ganie, 2014; Yadav et al., 2014). MicroRNAs are 21 to 24 nucleotide long RNA sequences derived from single-stranded RNA precursors that can form intra-molecular complementary hairpin structures. These molecules have significant regulatory potential of gene expression at the genetic and epigenetic level. They play a key role in plant genome response to (a)biotic stress factors. Especially, deeply conserved miRNA families are integral components of developmental processes in plant organism (Xie et al., 2010; Bej and Basak, 2014).

The objectives of this study were to verify the applicability and species transferability of miRNA-based markers and to identify genotype-specific polymorphism profiles for genetic diversity characterization of selected gladiolus genotypes.

Material and methodology

Biological material

Nine cultivars of hybrids gladiolus (*Gladiolus* × *gandavensis* Van Houtte) of interesting flower colours and shapes were analysed (Figure 1). All cultivars, except ‚Dandy' which comes from locality Kotešová (Slovak Republic), come from the town district Levice, locality Géňa (Slovak Republic). Detailed cultivars characterization is described in Table 1 and 2.

Genomic DNA was isolated from leaves (pooled sample of 5 randomly collected plants in the stage of flowering) by CTAB extraction procedure (Rogers and Bendich, 1994) and quantify by nanophotometer P360 (Implen). After spectrophotometric quantification was DNA diluted to 100 ng.μL^{-1}. The sequences of microRNA-based primers are shown in Table 3. The miRNA-based assay including results analyses was conducted based on Ražná et al. (2015).

Figure 1 Flowers morphological diversity of *Gladiolus* × *gandavensis* Van Houtte cultivars
A – Dandy; B – Falling Snow; C – Athos; D – Petra; E – Pulchritude; F – Fidorka; G – Správna Susane; H – Rajathos; I – Ráj Srdce. (Photo by M. Majtán)

Table 1 Origin characterization of gladiolus genotypes

Code	Cultivar	Introduction	Breeder	Pedigree
445	Správna Susan	2005	Belička	Gay Festival × seedling
341	Rajathos	2011	Belička	Raj Srdce × Athos
373	Pulchritude	1992	Klutey	Regency × Sabre × Apollo × Powder Puff
500	Falling Snow	1992	Mackenzie	Cliffs Of Dover × Incomparable
327	Petra	2001	Šaran	Elen × Red Alert
401	Ráj Srdce	2006	Mimránek	Pulchritude × Cream De Mint
401	Athos	1985	Hajduček	Darienka × Orient × Shell Pink
377	Dandy	2010	Rýpar	Regency × Sestra Štěst
471	Fidorka	2008	Belička	Super High Brow × seedling

Notes: Description provided by the breeder Majtán (2022)
code – code of North American Gladiolus Council

Table 2 Morphological description of flowers of gladiolus cultivars

Cultivars	Description
Správna Susan	base colour – medium salmon pink, dark red eye with attractive yellow frosted border; attractive unusual flower colouring, contrast of colour and petal shape – star-shaped flowers; flower diameter 11–14 cm, large-flowered, weak average flower set averaging 16 buds; medium maturity, 80–84 days; medium shrivelling; medium waxiness; unique cultivar due to the colour and shape of the flowers, but most susceptible of all to water stress – ear wilt
Rajathos	base colour – salmon pink, distinctive red-orange tongues with white edging; flower diameter 9.0–11.4 cm – medium flower size; medium maturity, 80–84 days; medium shrivelled with a star-shaped hint; strong waxiness; exceptional cultivar, regularly wins exhibitions in Slovakia, beautiful double ear row, average flower set of 26 flowers (can produce 34 flowers), one of the best flower sets ever – can have up to 12 flowers in bloom at a time, i.e. once as many as usual
Pulchritude	base colour – light lavender-olive; Dark olivaceous tongues on the lower three petals, only a subtle hint on the upper three; Flower diameter 9.0–11.4 cm – medium flower size; medium early, 75–79 days; weak shrinkage; medium waxiness; genotype bearing exceptional show parameters, excellent ear structure, average flower set of 22–26 buds, regular double row of flowers in the tall ear, in history often used for breeding new genotypes
Falling Snow	basic colour – white; flower diameter 9.0–11.4 cm – medium flower size; medium maturity, 80–84 days; medium shrivelling; medium waxiness; classic white cultivar, bearing medium characteristics, average flower set of 17 buds; mainly used for market purposes
Petra	basic colour – red; attractive unusual shading of flowers; flower diameter 9.0–11.4 cm – medium flower size; medium late, 85–90 days; strong shrinkage; strong waxiness; genotype with exceptional exhibition parameters, excellent ear structure, average flower set of 22–26 buds, often used in breeding because of its excellent exhibition parameters, regular arrangement of flowers in the ear, massive ear resistant to transport
Ráj Srdce	basic colour – white with a distinctive orange-red eye; unusual flower colouring, colour contrast; flower diameter 11–14 cm, large-flowered; medium-late, 85–90 days; medium shrivelling; high waxiness; cultivar used in breeding for its solid and multiple inflorescences (22–24 buds on average)
Athos	basic colour – white; orange-red strong colouring of the lower 3 petals; flower diameter 11–14 cm – large-flowered; medium early cultivar flowers in 80–84 days; medium shrivelling; strong waxiness; cultivar used in breeding for its interesting colour – distinctive orange-red colour of the petal tongues; weaker flower set with an average of 18 buds
Dandy	basic colour – lavender-purple to purple; flower colour – a combination of dark purple and creamy yellow; flower diameter 9.0–11.4 cm – medium flower size; medium late, 85–90 days; medium shrivelling; high waxiness; cultivar with exceptional show parameters, excellent ear structure, average flower set of 24 buds, not susceptible to transport due to its high wax content, good marketability
Fidorka	base colour – pale lavender-lavender; unusual flower colour, distinctive lilac tongue with vanilla edging; flower diameter 11–14 cm, large-flowered; medium maturity, 80–84 days; strong shrinkage with star-shaped petals; medium waxiness; genotype bearing above average show parameters, spikelet structure is more susceptible to irrigation conditions, if not regular may cause inflorescence curvature; average flower set of 22 buds

Note: Description provided by the breeder Majtán (2022)

Table 3 Sequences of applied miRNA-based markers

Primer	Sequences 5'–3'	Sequence origin
cca_miR156 – forward	TGA CAG AAG AGA GTG AGC AC	*Cynara cardunculus* L.
cca_miR156_reverse	GTG CTC ACT CTC TTC TGT CA	
ghr_miR156 – forward	AGG GAG GTG ACA GAA GAG AGT	*Gossypium hirsutum* L.
ghr_miR156_reverse	TGA GCA CGC AGA GCT TCA A	
hyp_miR156 – forward	TTG AGA GGG AGA GGG AAT TT	*Hypericum perforatum* L
hyp_miR156_reverse	TTG AAG GTG ATG ACA GAA GC	
ghr_miR160 – forward	TGG CTC CCT GTA TGC CAT TT	*Gossypium hirsutum* L.
ghr_miR160_reverse	TGG CTC CTC ATA CGC CAT TC	
mdo_miR160 – forward	TGC CTG GCT CCC TGT ATG CCA	*Malus domestica* Borkh.
mdo_miR160_reverse	TGG CAT ACA GGG AGC CAG GCA	
mdo_miR398 – forward	TGT GTT CTC AGG TCA GGG GTT	
mdo_miR398_reverse	AAC CCC TGA CCT GAG AAC ACA	
lus_miR408 – forward	GGC TGG GAA CAG ACA GAG CAT GGA	*Linum usitatissimum* L.
lus_miR408_reverse	GGG AAA AAG GCC AGG GAA GAG	
hyp_miR414 – forward	AGA GTA CAG GGA AAT GGA GGA	*Hypericum perforatum* L.,
hyp_miR414_reverse	CAC AGC GAA ACC CAC GAG	
gb_miR482 – forward	TGG GTT GTA GTC TTC AGG AGT GGG	*Ginkgo biloba* L.,
gb_miR482_reverse	GAA GGC AAT AGG AAT GGG AGG ATC	

Results and discussion

Phenotypic variability among the *Gladiolus* cultivars is extensive and is caused by genetic, environmental, and physiological factors (Singh et al., 2017b). Due to high conservation of miRNA sequences was developed an effective type of molecular markers useful not only for genetic diversity studies but also for functional polymorphism analysis. The fundamental potential of miRNA-based markers relies on the primers design based on the mature miRNAs sequences as a part of their step-loop structure. The advantages of this marker system include reproducibility and transferability across species (Fu et al., 2013). The high level of transferability demonstrates the usability of miRNA-based markers for comparative genome mapping and phylogenetic studies (Yadav et al., 2014).

Due to the unavailability of sequences of a given species or family, we used different types of miRNA-based markers whose amplification efficiency and species transferability have been verified in our previous studies. As these markers are derived from conserved miRNA sequences, a high degree of universality between genera is expected (Yadav et al., 2014). Here we applied 9 types of miRNA-base markers derived from sequences of conservative microRNA families (miR156, miR160, miR398, miR408, miRr414 and miR482) of following species: *Cynara cardunculus* L., *Ginkgo biloba* L., *Gossypium hirsutum* L., *Hypericum perforatum* L., *Linum usitatissimum* L. and *Malus domestica* Borkh.

Out of nine miRNA-based markers, only marker hyp-miR156, was not amplified and two markers, ghr-miR156 and ghr-miR160 amplified one monomorphic DNA fragment. Due to the standardization of DNA quantity and amplification conditions, different intensities of amplified miRNA locus can reflect the developmentally specific activity of these two types of markers (Figure 2). The family miR156 is one of the most stable and highly expressed miRNA families in plants (Xie et al., 2012), participating in regulation of plant growth and development and flowering time (Rubio-Somoza and Weigel, 2011). Stonger amplification of ghr-miR156 loci was observed in genotypes Athos, Pulchritude, Rajathos and Ráj Srdce. In terms of ancestry, these genotypes are strongly linked (Table 1). MiR160 family participates in meristematic tissue formation, differentiation, cell division and hormonal control (Vionnet, 2009). They also play roles in plant responses to diverse environmental factors (Shen et al., 2015). These miRNA-based loci did not show the same level of amplification as the previous ones, which may be related to the developmental phase of growth, as genotypes were sampled at the time of flowering, where differentiation and elongation were

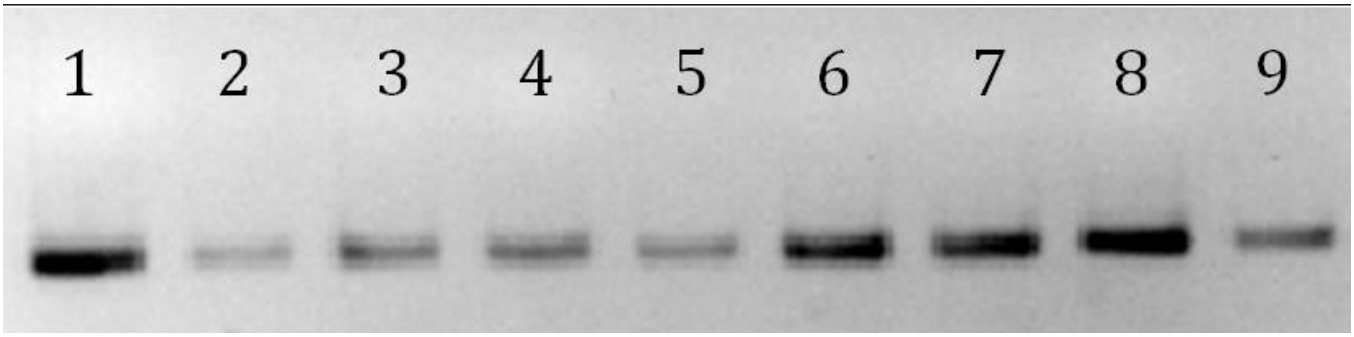

Figure 2 Amplification of ghr-miR156 locus in tested gladiolus cultivars
1 – Athos; 2 – Dandy; 3 – Falling Snow; 4 – Fidorka; 5 – Petra; 6 – Pulchritude; 7 – Rajathos; 8 – Ráj Srdce; 9 – Správna Suzanne

finalized. The role of miR156 in plant growth processes has also been confirmed by Hlavačková et al. (2016), where the proportion of the miR156 loci gradually increased depending on the stage development of the flax plants.

Monomorphic miRNA loci were amplified also by marker, mdo-miR398, where polymorphism was observed only in genotype Správna Suzane (picture not shown). This is a unique genotype due to the colour and shape of the flowers, but it is the most susceptible to water deficit, manifested by the conduct of the flower spikes, of all analysed cultivars. MiRNA398 is considered as stress-responsive miRNA involved in plant stress regulation mechanism (Zhu et al., 2011) and has been reported to be associated with various stress conditions as oxidative stress (Sunkar et al., 2006), water deficit (Trindade et al., 2010), salt stress and abscisic acid stress (Jia et al., 2009). Therefore, observed miR398 polymorphism in this gladiolus genotype may reflect the genome susceptibility to this abiotic stress.

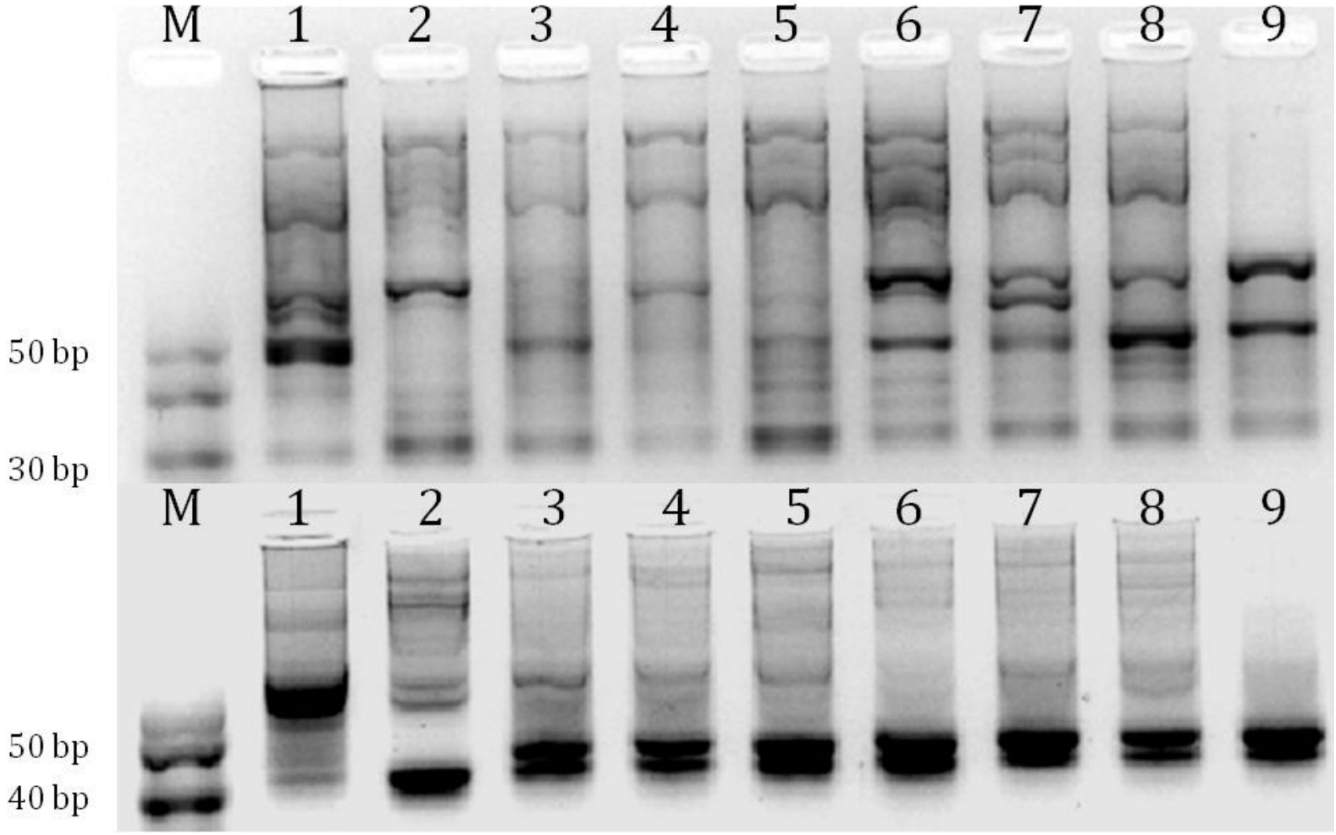

Figure 3 Amplification profile of lus-miR408 (upper) and hyp414 (lower) loci in tested gladiolus cultivars
1 – Athos; 2 – Dandy; 3 – Falling Snow; 4 – Fidorka; 5 – Petra; 6 – Pulchritude; 7 – Rajathos; 8 – Ráj Srdce; 9 – Správna Suzanne

In terms of amplification activity, we can sort the markers in descending order as follows, lus-miR408 (72 miRNA-based loci), hyp-miR414 (62 miRNA-based loci), cca-miR156 (54 miRNA-based loci), mdo-miR160 (47 miRNA-based loci), gb-miR482 (36 miRNA-based loci) and mdo-miR398 (20 miRNA-based loci).

Genotype-specific amplification profiles were observed by markers lus-miR408 and hyp-miR414 (Figure 3). These markers provided the highest number of amplified loci, with the average number of loci per genotype, 8 (lus-miR408) or 7 (hyp-miR414) respectively. Based on miRNA-based DNA fingerprinting is possible to distinguished individual genotypes profiles (Figure 4). Activity of miR408 underlies higher tolerance to salinity, cold, oxidative stress, drought, and osmotic stress (Ma et al., 2015). Constitutive expression of miR408 affects various stages of development and promotes intense plant growth and seed yield by increasing the efficiency of photosynthesis. Therefore, miR408 is likely to have a pleiotropic effect on plant growth and development (Pan et al., 2018).

Family miR414 play an essential function in plant growth, development, physiological and morphological changes, metabolism, and plant defense responses (Guleria and Yadav, 2011). In the genome of most analysed gladiolus cultivars (Falling Snow, Fidorka, Petra, Pulchritude, Rajathos, Ráj Srdce and Správna Susane) were amplified among others, two distinctive loci within the size interval from 40 bp up to 50 bp (Figure 3). In two genotypes were these loci amplified out of this size range, in genotype Anthos was the locus length approximately 70 bp and in genotype Dandy 40 bp.

The loci of marker cca-miR156 were amplified but without the observed polymorphism (picture not shown), so it is not a suitable marker for gladioulus diversity recording purposes. In the cultivar Fallin Snow, was not recorded the gb-miR482 loci amplification even after repeated amplification. MiR482 is involved in defence's mechanism of plants genome (Wang et al., 2015). Additional analyses would be needed to clarify the reasons for the absence of this locus; at this stage, the explanation would be speculative.

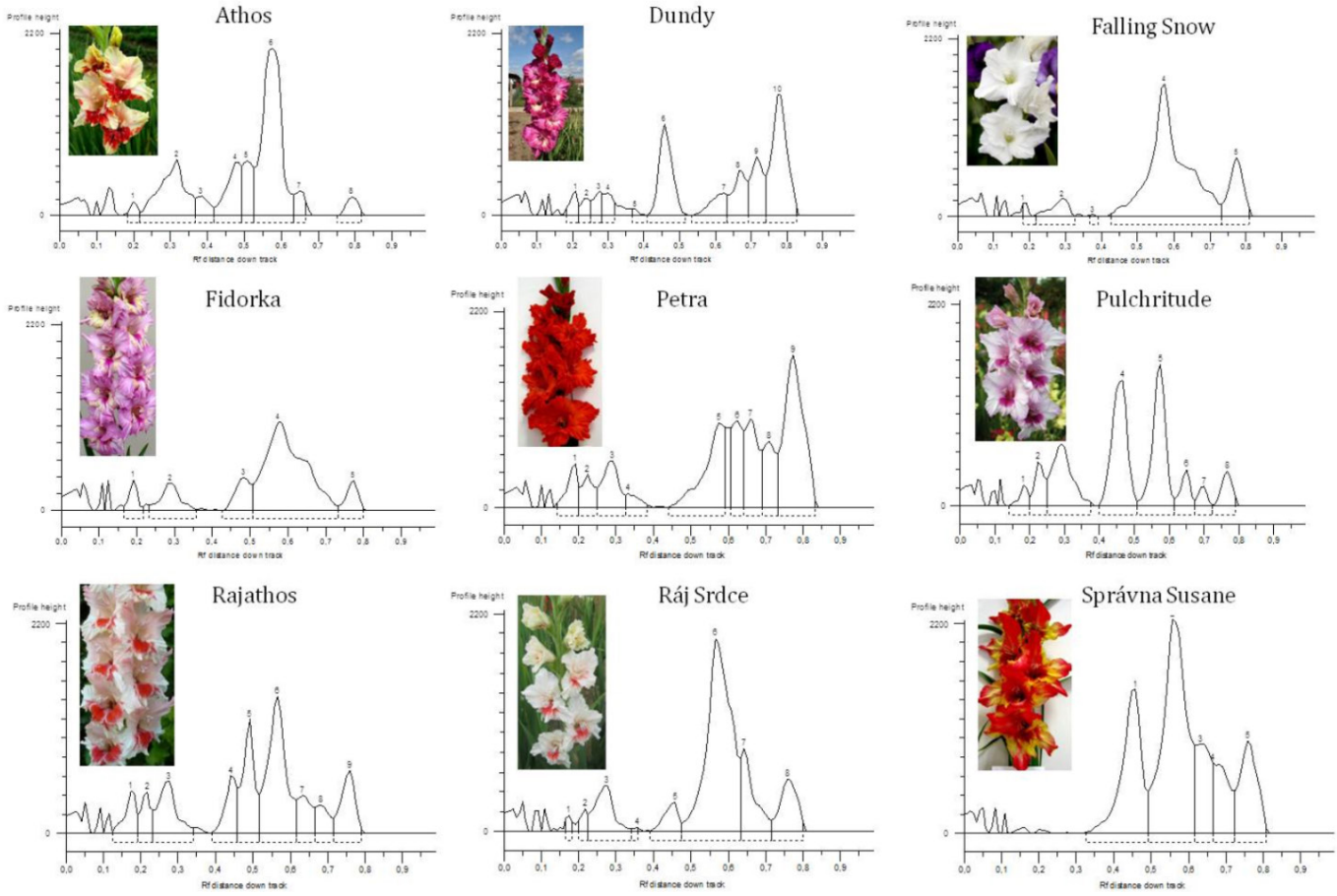

Figure 4 Elecrophoretic miRNA loci profile of lus-miR408 in tested gladiolus cultivars
1 – Athos; 2 – Dandy; 3 – Falling Snow; 4 – Fidorka; 5 – Petra; 6 – Pulchritude; 7 – Rajathos; 8 – Ráj Srdce; 9 – Správna Suzanne
(Photo of gladiolus by M. Majtán)

The evaluation of diversity in gladiolus genotypes based on phenotypic variability has its well-established platform (Momin et al., 2017; Patil et al., 2017; Ramzan et al., 2016) and irreplaceable place given the economic importance of this species. The purpose of several genetic studies was to characterize and identify the genetic diversity of the *Gladiolus* species by morphological and physiological markers (Singh et al., 2017a), minisatellite markers (Singh et al., 2017a; Singh et al., 2018) ISSR (Kumar et al., 2016; Chaudhary et al., 2018; Singh et al., 2017b; Singh et al., 2018), RAPD (Pragya et al., 2010), RAPD-derived SCAR markers (Singh et al., 2017b) and AFLP fingerprinting (Kutlunina et al., 2017). Singh et al. (2017) conducted a study focused on the analysis of the nucleotide diversity of gladiol, phylogeny of varieties and molecular systematic of the family Iridaceae using chloroplast DNA (cpDNA) regions.

One approach to assessing genetic potential is not only the application of DNA markers, but also the search for new types of functional molecular markers. Molecular markers based on miRNA molecules represent such type of functional markers (Fu et al., 2013; Mondal and Ganie, 2014; Yadav et al., 2014). Functional markers applied in this study correspond to miRNA sequences, particularly to precursor stem-loop regions, which are relatively highly conserved within closely related species (Fu et al., 2013; Yadav et al., 2014). The specificity of the molecular miRNA markers and reproducibility are improved by using higher annealing temperatures (primer binding), above 55 °C, during amplification in the "touchdown" PCR reaction.

For the genetic diversity analysis of gladiolus genotypes (*Gladiolus* × *gandavensis*), we applied markers which microRNA sequences are integrated in regulation of different processes in plants. Molecule's miRNAs are responsible for regulation of several developmental processes, including leaf morphology and plant polarity, root formation, processes of transition from embryogenic phase into vegetative, flowering time, formation of flower organs and reproduction and defense mechanisms (Xie et al., 2010; Cuperus et al., 2011; Chen et al., 2013; Hong and Jakson, 2015). Among others, the abundance of mature miRNAs, linked to the expression of MIRNA genes, varies greatly depending on miRNA family, tissue types or developmental stages.

Conclusions

Using molecular markers for genomic characterization of gladiolus cultivars provides a tool for selection of elite genetic resources for breeding process. Our study verified the suitability and species transferability of miRNA-based markers for genetic diversity characterization of selected gladiolus genotypes and identify genotype-specific functional markers originated from sequences of regulating microRNA molecules. Besides that, the application of functional miRNA-based markers will contribute to the deepening of knowledge about genomic polymorphism background of gladiolus.

Conflicts of interest

The authors declare no conflict of interest.

Ethical statement

This article does not contain any studies that would require an ethical statement.

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